# Evidence for a 5(6)-epoxytetraene intermediate in the biosynthesis of lipoxins in human leukocytes

## Conversion into lipoxin A by cytosolic epoxide hydrolase

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The existence of a 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid intermediate in the biosyntheses of lipoxins A and B has recently been proposed. In the present study, human leukocytes were exposed to 15-HETE and the divalent cation ionophore A23187 and alcohol trapping studies were performed. The products containing alkyltetraenes were isolated and characterized. HPLC analysis, UV spectroscopy and GC/MS of the products showed that 5,15-dihydroxy-14-*O*-alkyleicosatetraenoic acids were formed, indicating that 5(6)-epoxytetraenes (precursor of the trapping product) were formed in human leukocytes. To gain further evidence for the role of 5(6)-epoxytetraene intermediate in the biosynthesis of lipoxins, (15)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid was prepared by total chemical synthesis. When added to purified human liver cytosolic epoxide hydrolase, the epoxide was rapidly and quantitatively converted into LXA. The results provide further evidence for the role of a 5(6)epoxytetraene intermediate in the biosynthesis of lipoxins.

*Arachidonic acid* *Conjugated tetraene* *Lipoxygenase interaction product* *(Human)* *Leukocyte* *Epoxide*

## 1. INTRODUCTION

The oxygenative metabolism of arachidonic acid appears to play an important role in a wide variety of inflammatory responses (cf. [1]). Of the major lipoxygenases in mammalian tissues (i.e. 5-, 12- and 15-lipoxygenases), the 5- and 15-lipoxygenases are highly active metabolic pathways in human leukocytes [2]. Recently, interactions between the 5- and 15-lipoxygenases have been found to lead to the formation of a novel series of eicosanoids [3,4]. The series contains a conjugated tetraene structure as a characteristic feature and has been termed the lipoxins [3,4]. Some of the lipoxins have been shown to possess biological activities. For example, in human neutrophils LXA stimulates superoxide anion generation [4] and both LXA and LXB inhibit the activities of human natural killer cells [5]. Moreover, LXA and LXB stimulate and modulate the enzymatic activities of isolated protein kinase C in vitro [6].

Recently, we have established the absolute stereochemistry of LXA and LXB as 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid and 5(*S*),14(*R*),15(*S*)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid, re-

*Abbreviations:* LX, lipoxin; 15-HPETE, 15(*S*)-hydroperoxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; GC/MS, gas chromatography-mass spectrometry; UV, ultraviolet; RP-HPLC, reversed-phase high-performance liquid chromatography; $Me_3Si$, trimethylsilyl

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spectively [7,8]. Together with the determination of the absolute stereochemistries of LXA and LXB, evidence was presented for the transformation of 15-HETE acid into a 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid intermediate [7]. In this paper, further evidence for the existence of a 5(6)-epoxytetraene intermediate and its further enzymatic conversion is presented.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Materials and procedures employed were essentially the same as described in [3,4].

### 2.2. *Cell preparation*

Human leukocytes were prepared from peripheral blood of healthy donors as in [9]. The preparations consisted of mixed populations of leukocytes where the neutrophil contribution represented >90%. Cells were washed twice and suspended in Dulbecco's phosphate-buffered saline, pH 7.45.

### 2.3. *Incubation conditions*

Leukocytes ($100 \times 10^6$ cells per ml, 20 ml) were warmed to 37°C in a shaking water bath. 15-HETE (70 $\mu$M) and the ionophore of divalent cations A23187 (2.5 $\mu$M) were added simultaneously in ethanol (final concentration <1%, v/v) and 10 vols of either acidic ethanol or methanol were added after 30 s incubation. The pH values of the alcohols were lowered to 1 with HCl prior to their addition to the incubations. The samples were neutralized 2 min after the addition of the alcohol [10].

### 2.4. *Extraction and purification*

Samples were centrifuged and the supernatants (20 ml buffer and 200 ml alcohol) were evaporated under reduced pressure until approx. 20 ml was left in the flasks. Next, the samples were extracted with diethyl ether and subjected to silicic acid column chromatography as in [10]. Ethyl acetate fractions from silicic acid chromatography were evaporated, treated with diazomethane, and dissolved in methanol. Samples were injected onto an RP-HPLC column (Altex, ultrasphere-ODS, dp 5 $\mu$m, 4.6 mm × 25 cm) and eluted with methanol:water (70:30, v/v) at 1 ml/min (UV detector set at 301 nm). Materials exhibiting the UV spectrum characteristic of tetraenes were collected separately, treated again with diazomethane, and injected onto a Nucleosil C18 column (dp 5 $\mu$m, 4.6 mm × 25 cm) which was eluted with methanol:water (70:30, v/v) at 1 ml/min (fig.1).

### 2.5. *Structure determinations*

UV spectra were recorded with a Hewlett-Packard 8450A UV/visible spectrophotometer with methanol as a solvent. Catalytic hydrogenations were carried out as in [10]. GC/MS was performed with a Dani 3800 gas chromatograph HR PRV-2CH equipped with a fused silica capillary column (0.32 mm × 20 m, Orion) SE-30, and 7070E VG analytical mass spectrometer. The energy of the electron beam was set at 22.5 eV and the oven temperature at 230°C. Mass spectra were recorded using selective ion monitoring at *m/e* 173.

### 2.6. *Purification of human liver cytosolic epoxide hydrolase*

A semipurified preparation of cytosolic epoxide hydrolase was obtained by column chromatography on DEAE-cellulose, phenyl-Sepharose and hydroxyapatite.

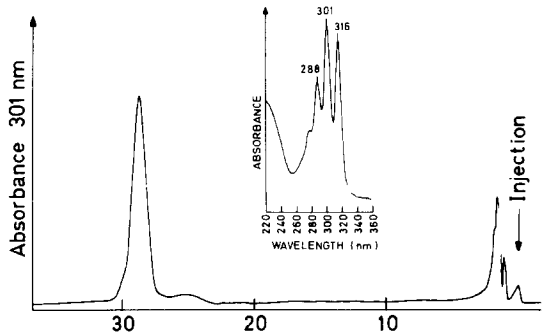



Fig.1. RP-HPLC chromatogram on Nucleosil C18 (methanol:water; 70:30, v/v) of trapping products treated with diazomethane from incubations of human leukocytes with 15-HETE and A23187. The material was previously purified by ether extraction, silicic acid column chromatography and RP-HPLC (Altex ultrasphere-ODS, methanol:water; 70:30, v/v). Inset: UV spectrum of eluted material in methanol.

### 2.7. *Incubation with the enzyme and extractions*

100 $\mu$l cytosolic epoxide hydrolase in argon-saturated 50 mM Tris-Cl (1.6 ng/ml, pH 8) was incubated at 37°C for 5 s with 50 $\mu$M 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid methyl ester added as an ethanol solution. Reactions were stopped with 2 vols MeOH. Samples were extracted twice with 10 vols diethyl ether. After evaporation under argon, extracted material was redissolved in 50 $\mu$l methanol. Samples were injected onto an RP-HPLC column (Altex-ultrasphere-ODS, dp 5 $\mu$m, 4.6 mm × 25 cm) and eluted with methanol:water (70:30, v/v) at a flow rate of 1 ml/min (UV detector set at 301 nm).

## 3. RESULTS

To provide further evidence for the existence of epoxytetraene intermediates in the biosynthesis of lipoxins alcohol trapping experiments were performed. Recent studies indicate that 15-HETE is also a precursor of LXA and LXB in activated human leukocytes [7,8]. Therefore, human leukocytes were incubated with 70 $\mu$M 15-HETE and 2.5 $\mu$M divalent cation ionophore A23187, and the incubations were stopped by addition of 10 vols acidic ethanol or methanol. This procedure partially eliminated the trapping of non-enzymatically derived products which may be generated from 15-HPETE. Moreover, the addition of 15-HETE excluded the formation of 14(15)-epoxides. Trapping with either ethanol or methanol led to the appearance of tetraene containing compounds which were less polar than either lipoxins A and B or their naturally occurring isomers. To achieve further purification before structure determination, the ethanol-derived trapping products were subjected to two different RP-HPLC systems (see fig.1). The UV spectrum of the material eluting with the retention time of 28–29 min showed a triplet of absorption bands at 288, 301 and 316 nm in methanol (see inset in fig.1).

To evaluate the structure of the trapping products obtained with ethanol, HPLC-purified material was converted to trimethylsilyl derivatives and analyzed by gas-liquid chromatography/mass spectrometry. The material eluted as a peak having an equivalent chain length corresponding to a *C*

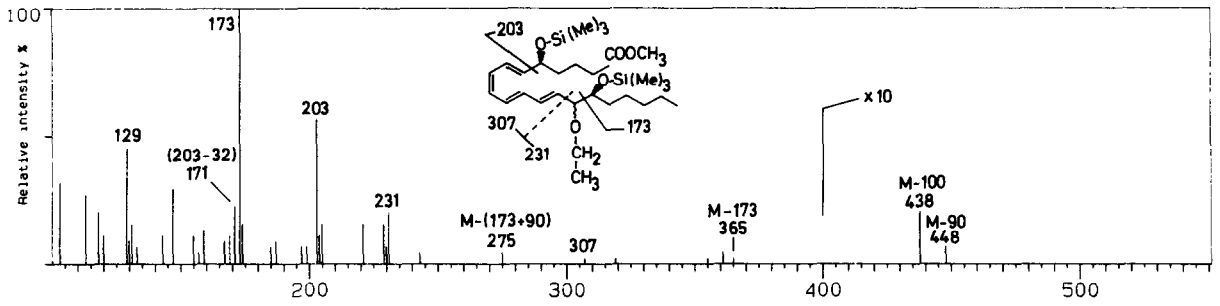



Fig.2. Mass spectrum of the $Me_3Si$ derivative of an ethanol trapping product.

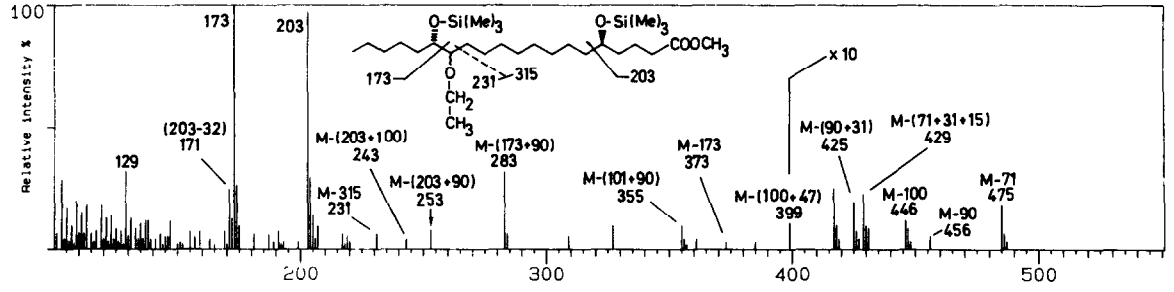



Fig.3. Mass spectrum at the $Me_3Si$ derivative of a catalytically hydrogenated ethanol trapping product.

value of 23.5 (SE-30). Prominent ions in its mass spectrum were observed at *m/e* 173 (base peak; $Me_3SiO^+ = CH(CH_2)_4\text{-}CH_3$), and 203 ($Me_3SiO^+ = CH\text{-}(CH_2)_3\text{-}COOCH_3$) (fig.2). Ions at lower intensities were observed at *m/e* 171 (203 − 32; elimination of $CH_3OH$), 231 $CH_3\text{-}CH_2\text{-}O\text{-}(Me_3SiO^+) = CH\text{-}(CH_2)_4\text{-}CH_3$, 275 (M − (173 + 90); loss of $Me_3SiO^+ = CH\text{-}(CH_2)_4\text{-}CH_3$ plus $Me_3SiOH$), 307 (M − 231), 365 (M − 173), 438 (M − 100; rearrangement followed by loss of $O = HC\text{-}(CH_2)_4\text{-}CH_3$, and 448 (M − 90; loss of $Me_3SiOH$).

To gain further evidence for the structure of the parent compound, we next examined the mass spectrum of the product formed upon catalytic hydrogenation (fig.3) (*C* value 25.2 on SE-30). Ions at high intensity were observed at 173 (base peak; $Me_3SiO^+ = CH\text{-}(CH_2)_4\text{-}CH_3$), 203 ($Me_3SiO^+ = CH\text{-}(CH_2)_3\text{-}COOCH_3$), 253 (M − (203 + 90); loss of $\cdot CH(OSiMe_3)\text{-}(CH_2)_3\text{-}COOCH_3$ plus $Me_3SiOH$), 283 (M − (173 + 90), loss of $Me_3SiO^+ = CH\text{-}(CH_2)_4\text{-}CH_3$ plus $Me_3SiOH$), 355 (M − (101 + 90); loss of $\cdot CH_2\text{-}(CH_2)_2\text{-}COOCH_3$ plus $Me_3SiOH$), 373 (M − 173), loss of $Me_3SiO^+ = CH\text{-}(CH_2)_4\text{-}CH_3$), 399, 425 (M − (90 + 31), loss of $Me_3SiOH$ plus $CH_3O$), 429 (M − (71 + 31 + 15); loss of $\cdot(CH_2)_4\text{-}CH_3$ plus $CH_3O$ plus $CH_3$), 446 (M − 100; rearrangement followed by loss of $O = HC\text{-}(CH_2)_4\text{-}CH_3$), 456 (M − 90; loss of $Me_3SiOH$), and 475 (M − 71; loss of $\cdot(CH_2)_4\text{-}CH_3$). The presence of these ions in the mass spectra obtained for the $Me_3Si$ derivative of the unsaturated compound and the product of hydrogenation are consistent with the assignment of the parent compound as 5,15-dihydroxy-14-*O*-ethyl-6,8,10,12-eicosatetraenoic acid.

To gain further evidence for the enzymatic nature of LXA formation, 15(*S*)-hydroxy-5,6-epoxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid was prepared by total synthesis (Nicolaou, K.C. et al., to be published) and was incubated with human liver cytosolic epoxide hydrolase. When added to epoxide hydrolase the epoxytetraene was rapidly and almost quantitatively converted into LXA (fig.4). In the absence of enzyme (i.e. buffer only) the 5,6-epoxytetraene hydrolyzed to a mixture of different lipoxin A and B isomers. In phosphate-buffered saline (pH 7.4), the half-life of the synthetic 5,6-epoxytetraene was less than 5 s as measured by a computer program with Hewlett-Packard 8451A diode array spectrophotometer.

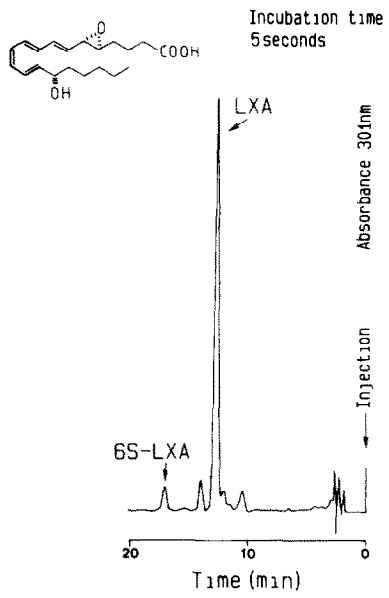



Fig.4. A model for the enzymatic formation of LXA: transformation of a synthetic 5,6-epoxytetraene by human liver epoxide hydrolase. RP-HPLC chromatogram on Altex ultrasphere-ODS (methanol:water; 70:30, v/v).

## 4. DISCUSSION

The lipoxins contain a conjugated tetraene structure and the complete stereochemistry of the biologically active compounds (i.e. LXA and LXB) has recently been assigned [7,8]. In accordance with the results of $^{18}O$ studies and the finding that 15-HETE is also a substrate for the biosynthesis of lipoxins A and B in activated human leukocytes it was proposed that once formed 5(*S*)-hydroperoxy-15(*S*)-hydroxyeicosatetraenoic acid is converted to a 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid intermediate [7]. This 5(6)-epoxytetraene may then serve as a common intermediate in both the enzymatic biosynthesis of LXA and LXB and the formation of non-enzymatic lipoxin isomers [7,8]. To obtain further evidence for the existence of a 5(6)-epoxytetraene, alcohol trapping experiments were performed with activated cells exposed to 15-HETE. Here, we report the isolation and the basic structure of the 15-HETE-derived 14-*O*-

alkyltetraene trapping products (e.g. 5,15-dihydroxy-14-*O*-alkyleicosatetraenoic acids).

Samples of the trapping products were obtained by ether extraction, silicic acid column chromatography, and chromatography on two different HPLC systems. Following HPLC the eluted material showed a triplet of absorption bands at 288, 301 and 316 nm (methanol) indicating the presence of a conjugated tetraene. The presence of two hydroxyl groups, an alkyl group, and four conjugated double bonds was established by GC/MS of the trimethylsilyl derivatives including the hydrogenated compound. In addition to the trapping product presented here some other tetraene containing trapping products were isolated (not shown).

The present results clearly demonstrate the formation of 5,15-dihydroxy-14-*O*-alkyleicosatetraenoic acid (upon addition of acidic alcohol) which in these studies serves as a marker of the 5(6)-epoxytetraene formation by leukocytes. Moreover, results obtained from the incubation of the synthetic 5,6-epoxytetraene with cytosolic epoxide hydrolase (fig.4) provide a clear model for the enzymatic formation of LXA. However, it remains to be investigated if a similar enzyme is involved in the formation of LXA in human leukocytes.

Others have also postulated the role of epoxytetraenes in the formation of lipoxins and related compounds [13]. Results of the present study provide evidence for the role of one possible 5(6)-epoxytetraene in the biosynthesis of lipoxins in human leukocytes. Taken together the results of these and previous studies [4,7,8] support the scheme of formation of LXA, LXB and their naturally occurring isomers presented in fig.5. Here, the 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid intermediate is transformed to LXB by attack at the C-14 position and generation of an 8-*cis* double bond. Alternatively, the 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid may be opened enzymatically by epoxide hydrolase into LXA. Other naturally occurring isomers may be formed by non-enzymatic hydrolysis at the 5(6)-epoxytetraene or either work-up or hemoprotein-catalyzed isomerizations [7,8].

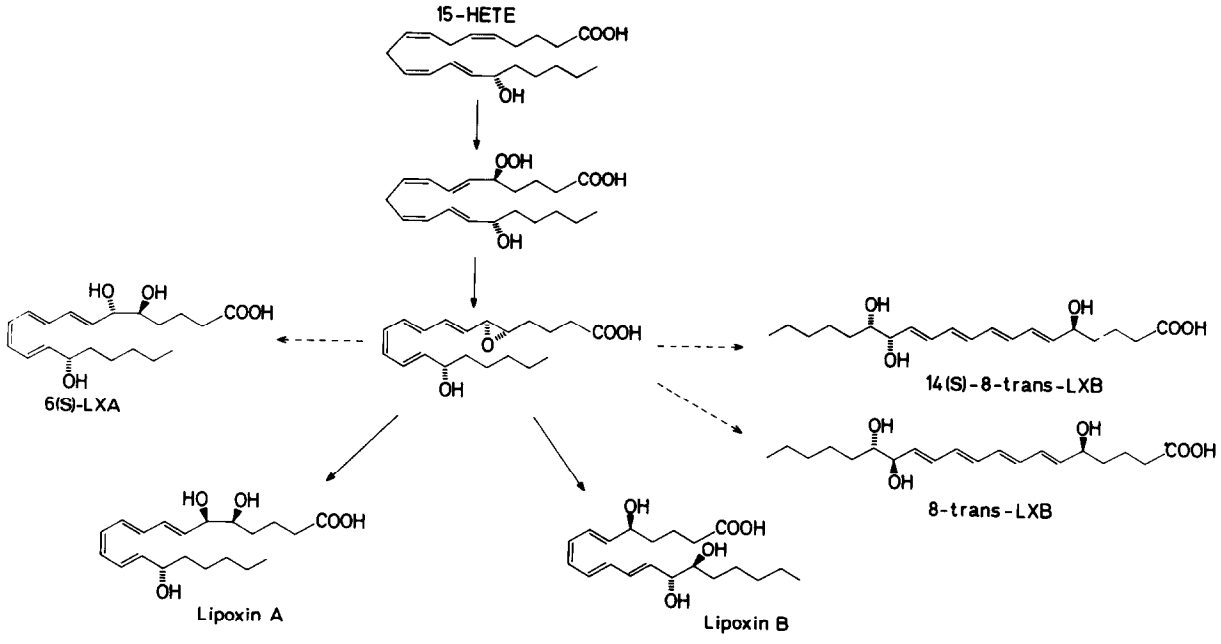



Fig.5. One possible biosynthetic pathway for lipoxin formation via a 15(*S*)-hydroxy-5,6-oxido-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid. Note that the stereochemistry of all of the compounds shown has been determined [7,8].

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